IMPACT OF VITAMIN D DEPLETION DURING DEVELOPMENT ON CC051 MICE

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ABSTRACT

Alison K. Homstad: Impact of vitamin D depletion during development on CC051 mice
(Under the direction of Folami Ideraabdullah)

Rodent models provide an ideal and reproducible environment to study vitamin D deficiency (VDD) during pregnancy. Our lab performed a series of reciprocal crosses with several strains of Collaborative Cross (CC) inbred mice to access the effects of developmental vitamin D deficiency (DVD) on the phenotypic outcomes, including adiposity, of the offspring. Using the CC mice allowed us to maximize the robustness of phenotypic responses to vitamin D deficiency. We identified a reciprocal cross, CC051 x CC041, that had disparate reactions to VDD during their development. The CC051 x CC041 offspring that were fed a low vitamin D (LVD) diet had more than two times as much fat mass as their control counterparts, while there was no significant difference in fat mass between the CC041 x CC051 mice on control and LVD diets. This promising data prompted us to investigate if we could see similar or stronger results in a CC051 x CC051 homozygous cross.

To my family and my lab, who saw me through tough times.
Thank you for everything

ACKNOWLEDGEMENTS

Throughout this study and my time at UNC, I have received a great deal of support and assistance. I would first like to thank my principle investigator, Dr. Folami Ideraabdullah, whose expertise was invaluable in formulating the study's research questions and methodology. Your insightful feedback buoyed my spirit and brought my work to a higher level. I would like to acknowledge my labmates in the Ideraabdullah lab for all of their amazing help. Dr. Jing Xue, I could not have done this without your impeccable training, guidance, and assistance. I am grateful to Liz Hutchinson and Changran Niu for their help in quantifying the calcium and insulin levels. I would also like to thank Edward Pietryk and Laetitia Meyrueix for assisting me in the lab or the mouse room whenever things got hectic! In addition, I would like to thank Dr. Jeff Sekelsky, Dr. Beverly Koller, and John Cornett for their wise counsel and sympathetic ear. You were always there for me. I have the deepest admiration for all of you! Finally, I could not have completed this dissertation without the support of my family. Thank you!

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LIST OF ABBREVIATIONS

CC	Collaborative Cross
CON	Control, Vitamin D sufficient
CYP	Cytochrome P450
DBP	Vitamin D Binding Protein
DOHaD	Developmental Origins of Health And Disease
DRIP	Vitamin D Receptor Interacting Protein
DVD	Developmental Vitamin D Deficiency
G_0	Generation 0
G_1	Generation 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GTT	Glucose Tolerance Test
HTF	Human Tubal Fluid
IU	International Unit
LVD	Low Vitamin D
PND	Postnatal Day
q-PCR	Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
UVB	Ultraviolet B
VDD	Vitamin D Deficient
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
VDRKO	Vitamin D Receptor Knockout
VDS	Vitamin D Sufficient
WAT	White Adipose Tissue

CHAPTER 1: INTRODUCTION

Vitamin D

Vitamins are organic compounds that are essential to health and are required in our diet because we cannot synthesize many of them in our bodies. While vitamin D is not an essential dietary factor, since our bodies can synthesize it, it is still essential for normal human physiological functioning. Vitamin D is a fat-soluble secosteroid that can be found in the body in two forms: Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol). Both of these forms of vitamin D can be obtained in the diet. Vitamin D_2 is found in plants and yeast, while Vitamin D_3 can be found in animal foodstuffs, fortified products, and supplements. However, only Vitamin D_3 can be produced within the human body via exposure to ultraviolet B (UVB) radiation. The process of vitamin D3 synthesis begins when 7-dehydrocholesterol present in the skin is irradiated with UVB rays from sunlight, causing it to photolyze into previtamin D_3. Previtamin D_3 will then undergo spontaneous isomerization to become vitamin D_3[1,2]. At this point, vitamin D_2 and D_3 are biologically inactive and are transported in the circulatory system via its major carrier protein, Vitamin D binding protein (DBP), in order to reach the liver[3]. Once in the liver, both forms of vitamin D undergo multiple hydroxylation steps by the P450 (CYP) enzymes with the hepatocytes to become biologically active. Both forms of vitamin D must undergo several hydroxylation steps by the P450 (CYP) enzymes to become biologically active. The first step in this process begins in the liver when CYP2R1 hydroxylates vitamin D's carbon 25 to form 25(OH)D (calcidiol)[4–6]. Then, the last step occurs when 25(OH)D reaches the kidney and CYP27B1 hydroxylates it at the 1α carbon position, thus creating the final active metabolite, $1,25(OH)_2D$ (calcitriol)[7–9]

The activated forms of vitamin D exit the kidney to be disseminated throughout the body to its target tissues via the bloodstream. Once within the cells of its target tissues, vitamin D binds to its specific nuclear hormone receptor, the vitamin D receptor (VDR)[10]. The vitamin D-bound VDR then forms a heterodimer with another nuclear hormone receptor called the retinoid X receptor (RXR)[11]. The VDR heterodimer can then enter the cell's nucleus to recruit the DRIP (D receptor-interacting protein) complex and other co-activators[12]. The VDR heterodimer within this complex can then bind to the Vitamin D Responsive Elements (VDRE) within the genomic DNA in order to regulate the expression of many different downstream gene products[13-15].

Vitamin D Deficiency and Obesity

Vitamin D has numerous and diverse roles in human physiology and cellular function. Historically, one of the most studied roles of vitamin D has been its involvement in calcium regulation within the body. However, vitamin D is increasingly being recognized for its emerging role in the developmental programming of adiposity[16-18], thus making vitamin D deficiency (VDD) during pregnancy a growing concern for public health advocates. At the moment, VDD is a worldwide problem that is defined as having a concentration of 25(OH)D <50 nmol/L[19] and affects populations at different rates; 7% in Southern Africa and up to 100% in some parts of Northern Europe[20]. The prevalence of VDD during pregnancy in the United States has been estimated to be in the range of 27-91%[20]. For the pregnant person, VDD during pregnancy is associated with numerous adverse health outcomes, such as preeclampsia and gestational diabetes, that can also affect offspring health[21-23]. While developmental vitamin D deficiency (DVD) in offspring has been associated with autoimmune disorders, low birth weight, insulin resistance, increased adiposity, and impaired skeletal and brain development[18,24-29]. In rodent studies, the damaging metabolic effects of DVD have been shown to continue into adulthood and are seen in adverse health outcomes like glycemic dysregulation, steatosis of the liver, blood hyperlipidemia, systemic chronic inflammation[27,30-33]. These types of metabolic dysfunction commonly seen in obese humans can increase the likelihood of developing type 2

diabetes mellitus, cardiovascular disease, and nonalcoholic fatty liver disease[34-38]. Thus, making understanding how DVD affects the developmental programming of adiposity paramount to understanding obesity from a developmental origin of health and disease (DOHaD) perspective.

Rodent models provide an ideal and reproducible environment to study VDD during pregnancy. Our lab performed a series of reciprocal crosses with several strains of Collaborative Cross (CC) inbred mice to access the effects of DVD on the phenotypic outcomes, including adiposity, of the offspring. Using the CC mice allowed us to maximize the robustness of phenotypic responses to vitamin D deficiency. We identified a reciprocal cross, CC051 x CC041, that had disparate reactions to VDD during their development. The CC051 x CC041 offspring that were fed a low vitamin D (LVD) diet had more than two times as much fat mass as their control counterparts, while there was no significant difference in fat mass between the CC041 x CC051 mice on control and LVD diets. This promising data prompted us to investigate if we could see similar or stronger results in a CC051 x CC051 homozygous cross.

Overall Objective

To evaluate the phenotypic outcomes of vitamin D deficiency during pregnancy in CC051 dams and during gestation and development in CC051 offspring.

Study Hypothesis

I hypothesize that dietary deficiency of Vitamin D during gestation and development will impact metabolic regulation leading to disparate phenotypic outcomes in CC051 mice compared to controls.

CHAPTER 2: METHODS

Animals: Housing, dietary treatment, and breeding scheme

Animal handling was humanely and ethically performed in accordance with the Guide for the Care and Use of Laboratory Animals under the animal use protocol at the University of North Carolina at Chapel Hill. The Collaborative Cross (CC) inbred strain CC051/TauUnc (CC051) mice were purchased from the UNC Systems Genetics Core Facility (Chapel Hill, NC). In the vivarium that the CC51 mice were housed, the temperature was maintained at 22°C with 50% humidity. The vivarium operated on a 12:12 hour light-dark cycle in which unfiltered fluorescent bulbs were the light source. Thus, all mice were exposed to UV B exposure (wavelength: 280–315 nm) at a rate of $8.39E^{-7}$ W/cm^2. Sterilized water and rodent chow were provided to the mice *ad libitum*.

Virgin CC051 mice (G_0 dams) were fed a standard rodent chow diet containing 2.0 IU vitamin D_3/g (Teklad 2020SX) until 14-15 weeks of age when they were randomly assigned either a purified vitamin D sufficient diet (CON; ENVIGO TD.89124) containing 2.2 IU vitamin D_3/g or a purified vitamin D deficient diet (Low vitamin D, LVD; ENVIGO TD.89124) containing 0 IU vitamin D_3/g. The G_0 dams began this dietary treatment 5 weeks before mating and remained on these diets throughout gestation, lactation, and weaning. All dams in this study were euthanized after pup weaning, and all offspring were transferred at weaning to a standard rodent chow diet of 2.0 IU vitamin D_3/g (Teklad 2020SX).

First-generation (G_1) offspring were generated via trio or harem breeding. G_1 mice were weaned at postnatal day (PND) 21 and matured to adulthood. A subset of G_1 neonates within 6 litters was euthanized for body composition measurements. All adult G_1 mice were euthanized at 12-13 weeks of age. Tissues and blood were harvested from all mice post-euthanasia.

Food consumption and body measurements

Consumption of experimental CON and LVD feed intake by G_0 dams was monitored weekly by food weight. Bodyweight measurements were taken weekly for both G_0 dams and G_1 mice throughout the study by balance. For G_1 mice, body composition was assessed at weaning (PND 28) and 12 weeks of age via EchoMRI by the Animal Metabolism Phenotyping Core (Chapel Hill, NC). Free water mass was subtracted from the bodyweight when calculating the fat and lean mass ratio. Crown-rump length, anogenital distance, and femur length were measured at euthanasia for all mice. The crown-rump length was also assessed at birth (PND 0) for all G_1 offspring. For G_1 neonates euthanized at PND 9 the weights of the body, liver, heart, and kidneys were measured.

Glucose Tolerance Testing

Glucose tolerance tests (GTTs) were performed for G_0 dams before dietary treatment began (day 0; D0), before mating (D35), and at weaning. The GTTs for G_1 offspring were performed at weaning (PND 28) and 12 weeks of age. For GTTs, mice fasted for 12 hours and glucose was injected intraperitoneally at 2g/kg per total body weight for G_0 dams and 2g/kg per lean body mass for G_1 offspring (determined via EchoMRI). A glucometer (AccuCheck) was used to measure blood glucose concentrations via tail sampling over 2 hours at 0, 15, 30, 45, 60, and 120 minutes.

Serum and Tissue Collection

All adult mice were euthanized via carbon dioxide exposure followed by cervical dislocation. Whole blood was collected without anticoagulant via submandibular bleed prior to euthanasia and cardiac puncture immediately after euthanasia. Serum was isolated from the blood collections via centrifugation and snap-frozen in liquid nitrogen.

The following tissues were removed, weighed via analytical balance, and snap-frozen in liquid nitrogen following euthanasia: Liver, kidneys, inguinal white adipose tissue (WAT), and testes/ovaries.

After being removed and weighed, the liver and the WAT were sectioned into pieces. The left and right medial lobes of the liver and a small section of the WAT were preserved for histology, while the remainder was snap-frozen in liquid nitrogen.

Isolation and quantification of sperm from G_1 male offspring

The intact cauda epididymis and vas deferens were removed after euthanasia and placed in Human Tubal Fluid (HTF) buffer (pH7.5, 100 mM NaCl, 5 mM KCl, 0.368 mM KH2PO4, 0.2 mM MgSO4, 2 mM CaCl2, and 5 mg/ml BSA) at 37°C. The epididymis and vas deferens were cut open under a dissection microscope. The opened epididymis and vas deferens were incubated for 10 min at 37°C to allow for mature sperm to swim out into the HTF buffer. The supernatant HTF buffer containing the sperm was carefully transferred to 1.5-ml tubes. A 1:50 dilution of the sperm resuspension was used for quantification by hemocytometer.

Quantification of insulin, calcium, and vitamin D metabolites

The G_0 dam submandibular serum collected before euthanasia was used for the quantification of insulin and calcium. Insulin and serum calcium concentrations were quantified using the UltraSensitive Mouse Insulin ELISA kit (CrystalChem) and the Calcium Colorimetric Assay kit (BioVision) according to manufacturers' instructions, respectively.

The G_0 dam cardiac puncture serum collected after euthanasia was used for the measurement of vitamin D metabolites. For samples in which there was not enough cardiac puncture serum, cardiac puncture serum and submandibular serum were pooled. The UC Davis Lipid Analytical Core Facility (Davis, California) measured the following vitamin D metabolites via mass spectrometry: $25(OH)D_2$, $25(OH)D_3$, $1,25(OH)_2D_2$, and $1,25(OH)_2D_3$. The vitamin D_2 and D_3 forms of both vitamin 25(OH)D and $1,25(OH)_2D$ were combined in statistical analysis of the metabolites.

Statistical Analyses

Statistical analyses were performed using JMP Pro software version 12.2.0 (SAS, NC). Linear regression was performed to determine the relationship between each phenotype and diet. Percent difference in body weight was calculated using the percentage difference formula:

$| V1 – V2 | / [(V1 + V2) / 2] * 100$

For the G_1 offspring, all analyses were separated by sex. For all comparisons, p-value <0.05 is considered statistically significant.

CHAPTER 3: IMPACT OF VITAMIN D DEPLETION ON CC051 G_0 DAMS

Maternal dietary depletion of 25(OH)D_3 does not affect mating outcomes

Vitamin D depletion in dams on the LVD diet did not lead to decreased fecundity (% of matings with a litter), fertility (litter size at birth), or postnatal viability (litter size at weaning and male/female ratio) (Table 1).

Table 1. Summary of G_1 breeding outcomes.

Diet	# of litters	% of matings with litter	Average litter size ± S.E.M.		Male percentage (mean ± S.E.M.)
			At birth	At weaning	
CON	5	41.67%	6.4±1.21	5.4±1.40	49.61±2.60
LVD	5	62.50%	6.8±0.8	5.4±1.03	42.55±6.15

Litter size at birth does not include litter sizes of zero (0). Litter size at weaning includes litters lost between birth and weaning.
S.E.M., standard error of the mean.

Dietary depletion of 25(OH)D_3 elicits no effect on calcium levels in adult CC051 dams

Serum was collected from the G_0 dams a week after pup weaning to quantify circulating sera 25(OH)D levels. The G_0 dams fed the diet lacking vitamin D (LVD) (Table 2) had significantly lower levels of 25(OH)D compared to the G_0 dams fed the control diet (CON) (p = 6.65 x 10^{-3}, Fig. 1b). Despite the lower levels of circulating sera 25(OH)D in the LVD dams, there were no significant differences in serum calcium and serum insulin levels between the diets (Fig. 1c-d).

Table 2. Overview of diets.

Cross	Diet	Treatment	Vitamin D IU/g
CC051xCC051	TD.89124	CON	2.2
	TD.89123	LVD	0

The dams on both diets were weighed weekly for 6 weeks from the start of the diet until breeding (Fig. 1a). While bodyweight increased for both LVD and CON dams over the 6 weeks, the LVD dams showed no significant difference in body weight compared to their CON counterparts each week (Fig. 2a). Additionally, there was no significant difference in food intake between the LVD and the CON dams (Fig. 2b).

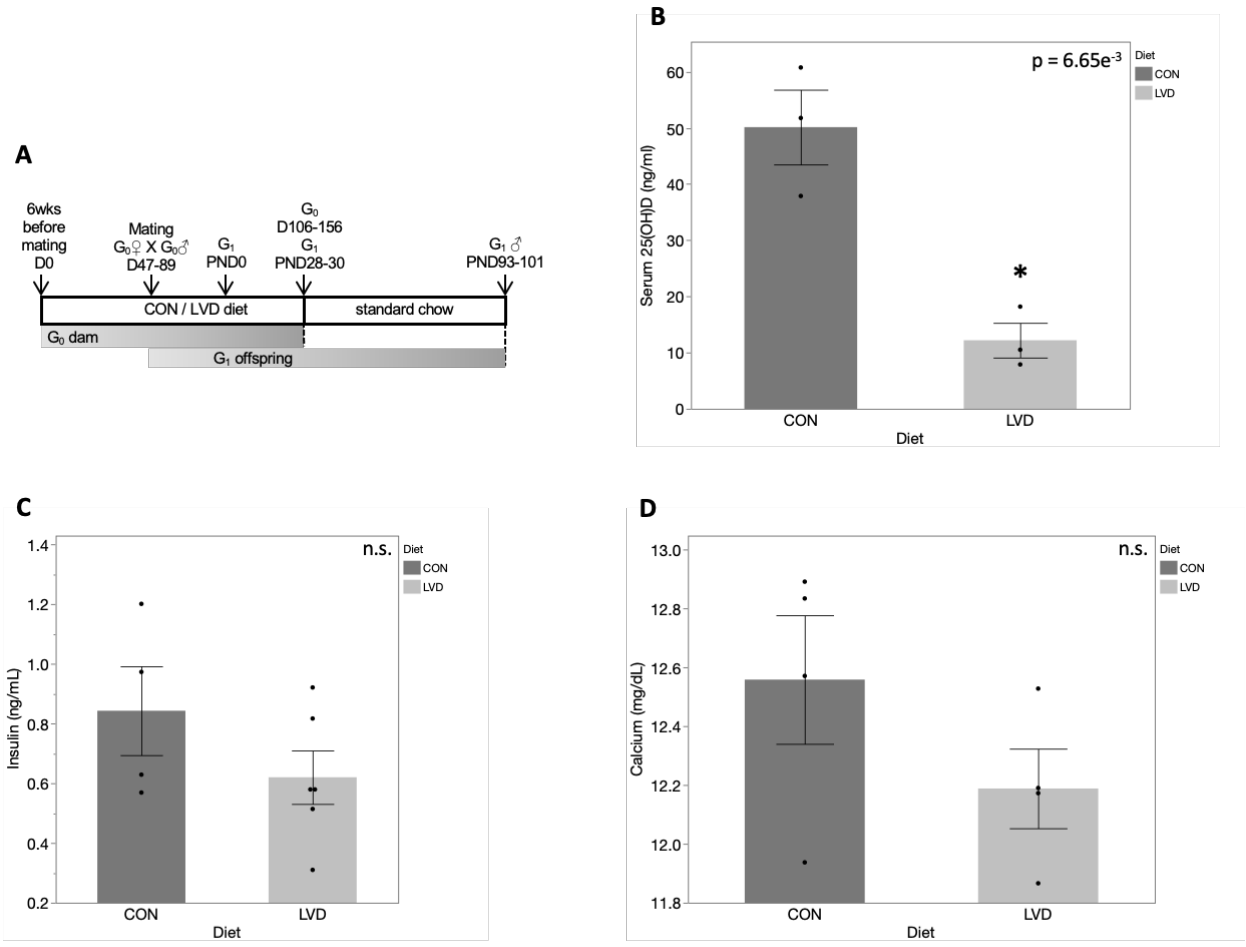


Figure 1. Study design and the effects of dietary depletion of vitamin D on CC051 G_0 dams Each dot represents a single mouse. (A) Study dietary treatment scheme. (B) Serum 25(OH)D measured by mass spectrometry for each diet. (C) Fasting insulin. (D) Circulating levels of calcium.

Dietary depletion of 25(OH)D_3 improves glucose tolerance of CC051 dams

Intraperitoneal glucose tolerance tests were performed on the G_0 dams at three timepoints: Pre-diet, Pre-mating, and Weaning of pups (Fig. 1a). As expected, there was no significant difference in baseline glucose tolerance between the G_0 dams that would be assigned either the CON or LVD diets (Fig. 2c-d, Pre-diet GTT). The LVD dams after 6 weeks on the diet exhibited a significant increase in glucose tolerance ($p = 4.45 \times 10^{-2}$, Fig. 2c, Pre-mate GTT at 60 min.; $p = 4.36 \times 10^{-2}$, Fig. 2d, Pre-mate GTT). While not significant, the blood glucose levels during the ipGTT at weaning continued to be lower for the LVD dams than the CON dams (Fig. 2c-d, Wean GTT).

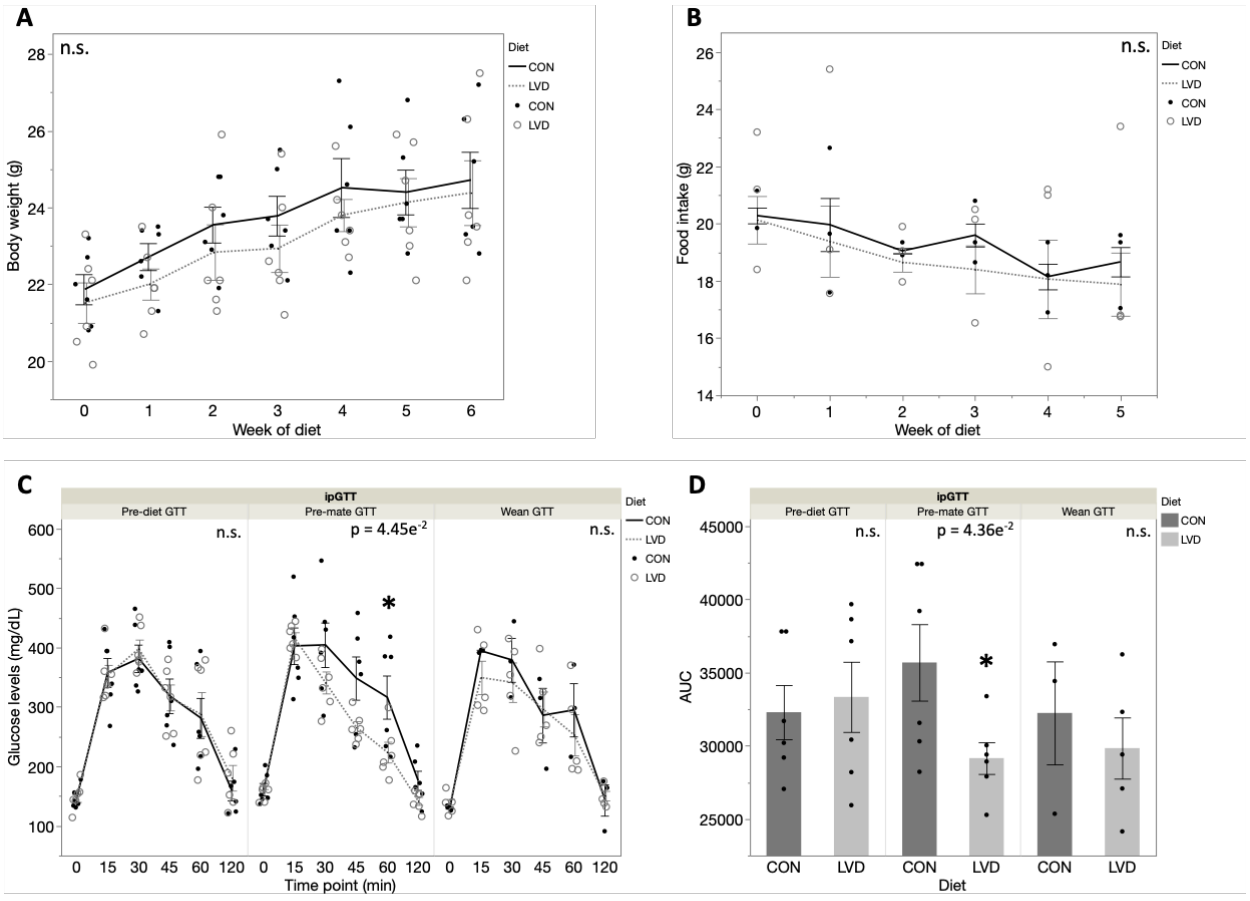


Figure 2. Effects of dietary depletion of vitamin D on CC051 G_0 dams continued Each dot represents a single mouse.
(A) Body weight for week 0-6 on diet. (B) Food intake for week 0-5 on diet. (C) ipGTTs and (D) AUCs at pre-diet, pre-mate, and wean timepoints.

CHAPTER 4: IMPACT OF DEVELOPMENTAL VITAMIN D DEFICIENCY ON CC051 G_1 OFFSPRING

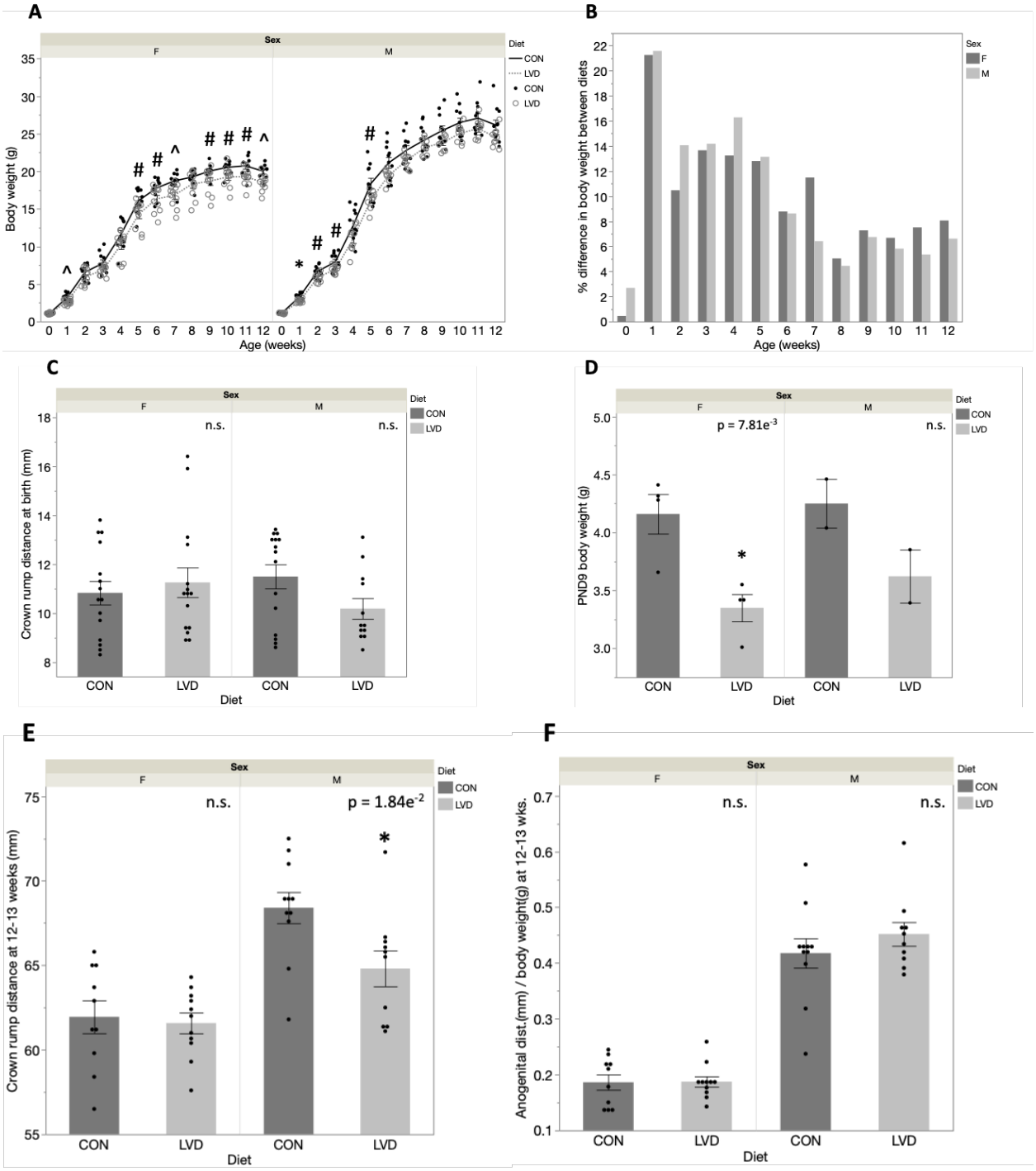
Maternal dietary depletion of 25(OH)D_3 caused post-natal growth retardation in CC051 G_1 offspring

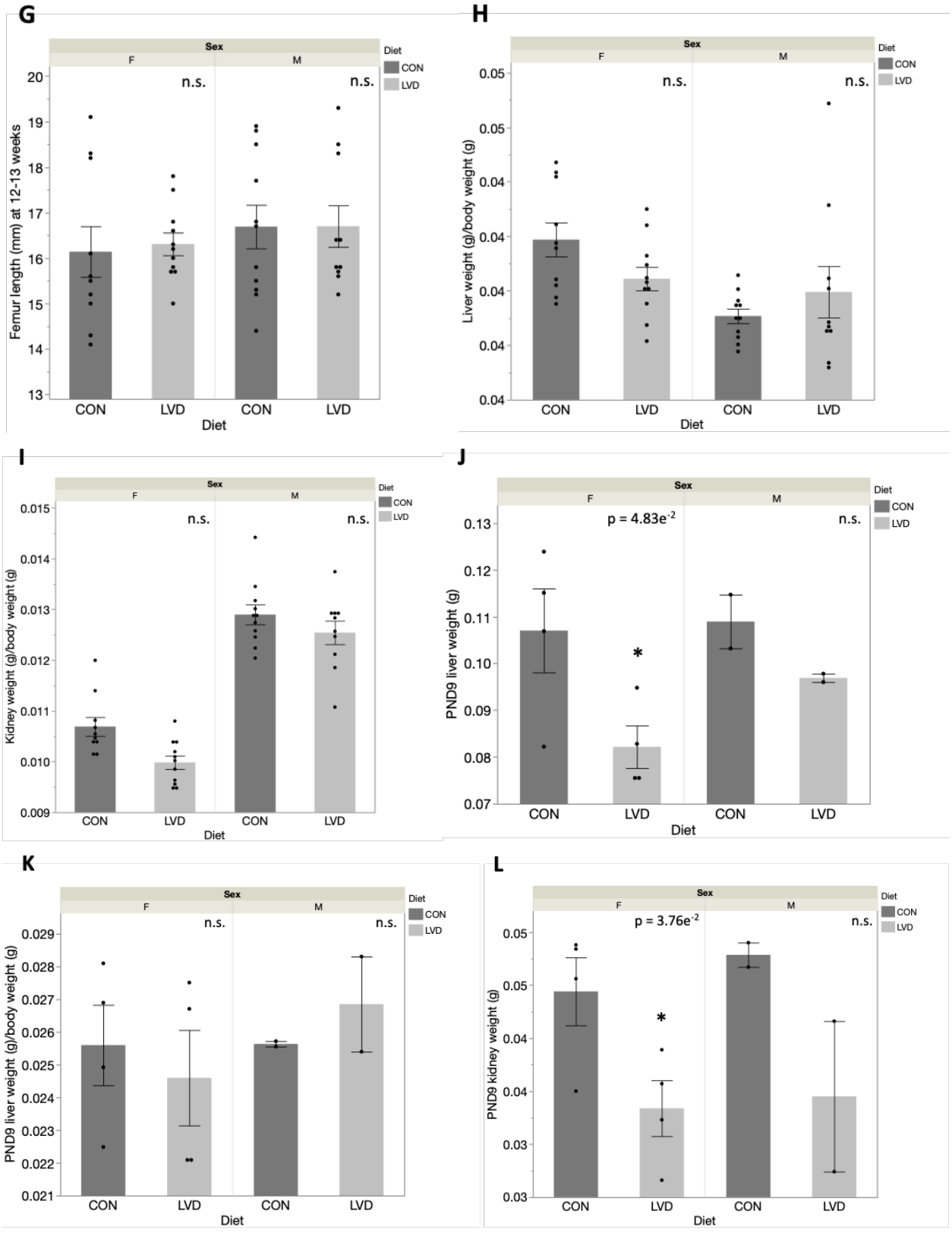
The G_1 offspring were weighed weekly for 12 weeks starting at their birth (Fig. 3a). G_1 LVD offspring tended to weigh less than G_1 CON offspring for both males and females. This difference in weights between G_1 CON and LVD offspring began to arise at week 1 (Fig. 3b) and was significant at several time points (Fig. 3a Males: $p = 6.05 \times 10^{-4}$, week 1; $p = 2.32 \times 10^{-2}$, week 2; $p = 3.53 \times 10^{-2}$, week 3; $p = 2.58 \times 10^{-2}$, week 5; Females: $p = 1.05 \times 10^{-3}$, week 1; $p = 2.48 \times 10^{-2}$, week 5; $p = 4.52 \times 10^{-2}$, week 6; $p = 4.33 \times 10^{-3}$, week 7; $p = 3.48 \times 10^{-2}$, week 9; $p = 2.54 \times 10^{-2}$, week 10; $p = 1.30 \times 10^{-2}$, week 11; $p = 2.85 \times 10^{-3}$, week 12) (Fig. 3d Females: $p = 7.81 \times 10^{-3}$, PND9).

The crown-to-rump distance was measured at birth and 12-13 weeks of age. There was no significant difference in crown-to-rump distance between G_1 CON and LVD offspring at birth (Fig. 3c). However, at 12-13 weeks of age, the G_1 LVD males were significantly shorter than the G_1 CON males ($p = 1.84 \times 10^{-2}$, Fig. 3e). The anogenital distance and femur length were measured only at 12-13 weeks of age for the G_1 offspring and no significant difference was found (Fig. 3f-g).

The liver and kidney organs were weighed at PND9 and 12-13 weeks of age. When adjusted for body weight, there were no significant differences between the G_1 CON and LVD offspring at both PND9 and 12-13 weeks of age for liver and kidney weight (Fig. 3h-l, 3k and 3m). However, the unadjusted liver and kidney weights of the G_1 LVD females were significantly lighter than the G_1 CON females at PND9 ($p = 4.83 \times 10^{-2}$, Fig. 3j, liver weight; $p = 3.76 \times 10^{-2}$, Fig. 3l, kidney weight). The heart was weighed only at PND9 for the G_1 offspring. Just as before,

there was no significant difference found for heart weight when it has been adjusted for bodyweight between the G_1 CON and LVD offspring of both sexes (Fig. 3o). Yet, the unadjusted heart weights of the G_1 LVD males were significantly lighter than their CON counterparts (p = 9.38 x 10^{-3}, Fig. 3n).




G
Sex
F
M
Diet
CON
LVD
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Femur length (mm) at 12-13 weeks
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19
18
17
16
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13
CON
LVD
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Diet
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n.s.
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Liver weight (g)/body weight (g)
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Kidney weight (g)/body weight (g)
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PND9 liver weight (g)
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PND9 liver weight (g)/body weight (g)
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p = 3.76e-2
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PND9 kidney weight (g)
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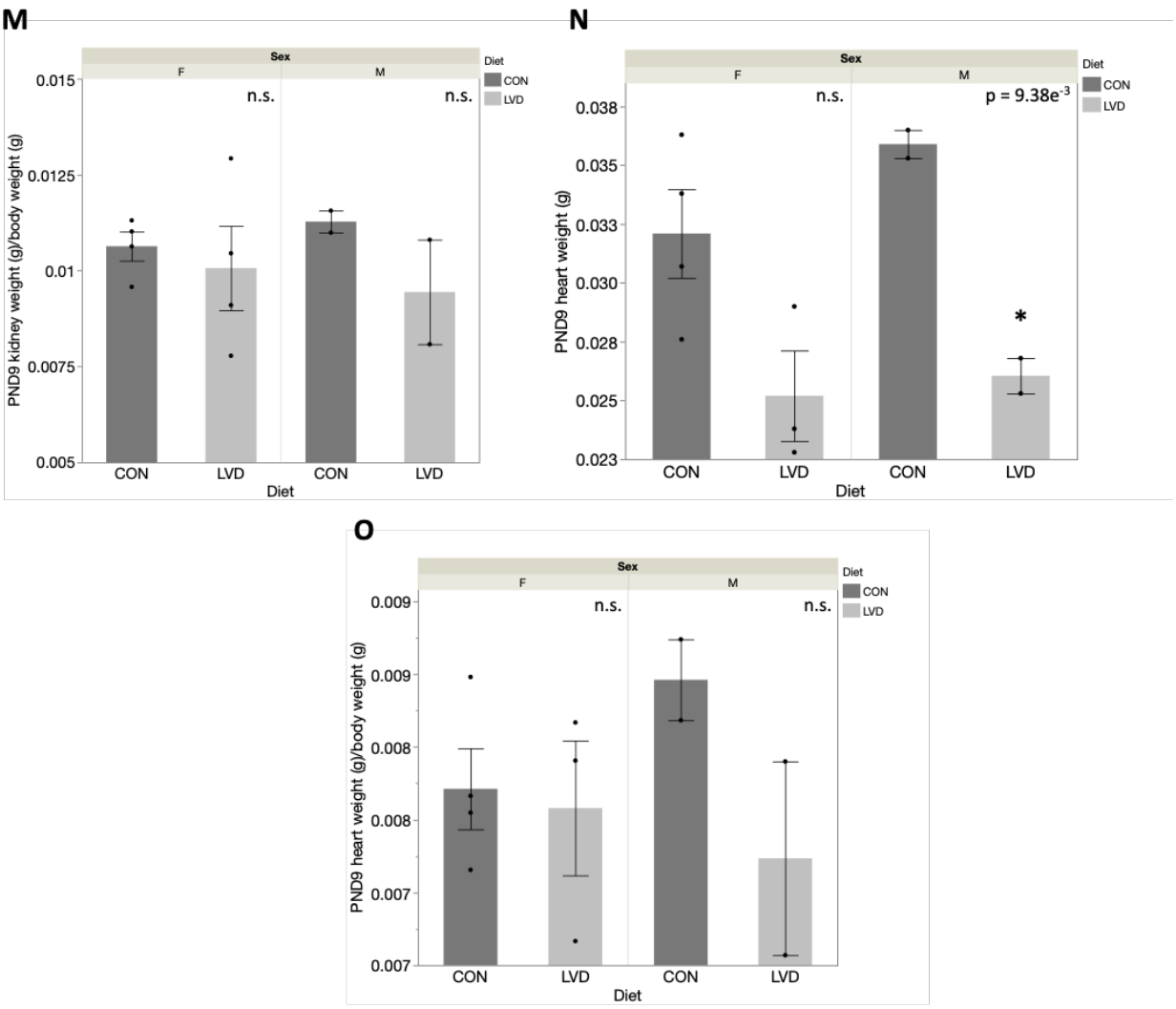


Figure 3. Effects of developmental vitamin D deficiency on CC051 G_1 offspring Each dot represents a single mouse. Data separated by diet and sex. (A) Body weight from 0-12 weeks. #p < 0.05, ^ p < 0.01, * p < 0.001. (B) % differences in body weight by week between diets. (C) Body weight at postnatal day 9 (PND9). (D) Crown rump distance at birth. (E) Crown rump distance, (F) anogenital distance, and (G) femur length at 12-13 weeks of age. (H) Liver and (I) kidney weight adjusted for body weight at 12-13 weeks of age. (J) Unadjusted and (K) adjusted liver weight at PND9. (L) Unadjusted and (M) adjusted kidney weight at PND9. (N) Unadjusted and (O) adjusted heart weight at PND9.

Maternal dietary depletion of 25(OH)D_3 does not affect CC051 G_1 body composition

The G_1 offspring had their body composition (% lean and fat mass) measured at 4 and 12 weeks of age. At both time points, the G_1 CON and LVD offspring did not differ in body composition (Fig. 4a-b). Additionally, there was no significant difference in perigonadal fat weight collected at 12 weeks of age between the G_1 CON and LVD offspring (Fig. 4c).

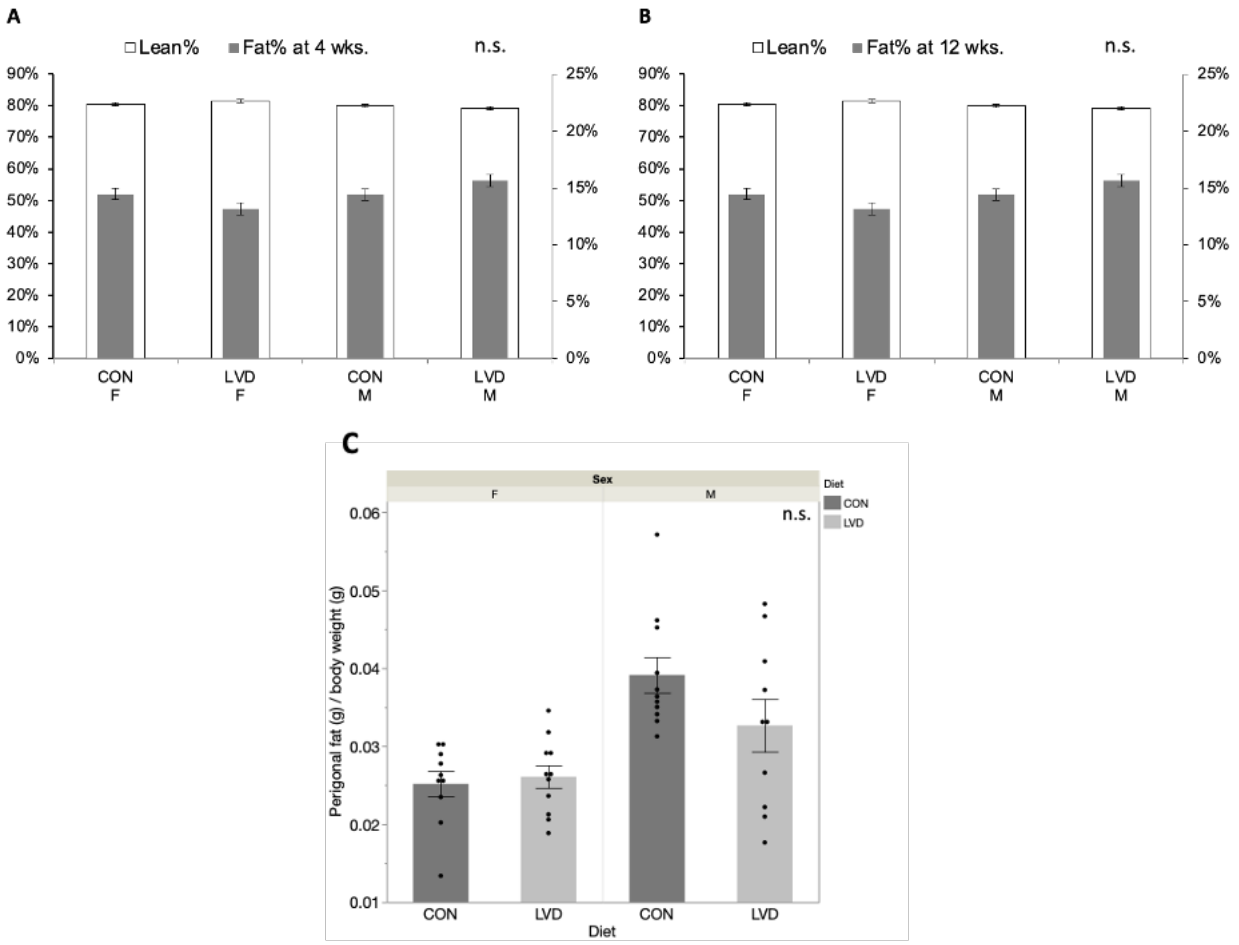
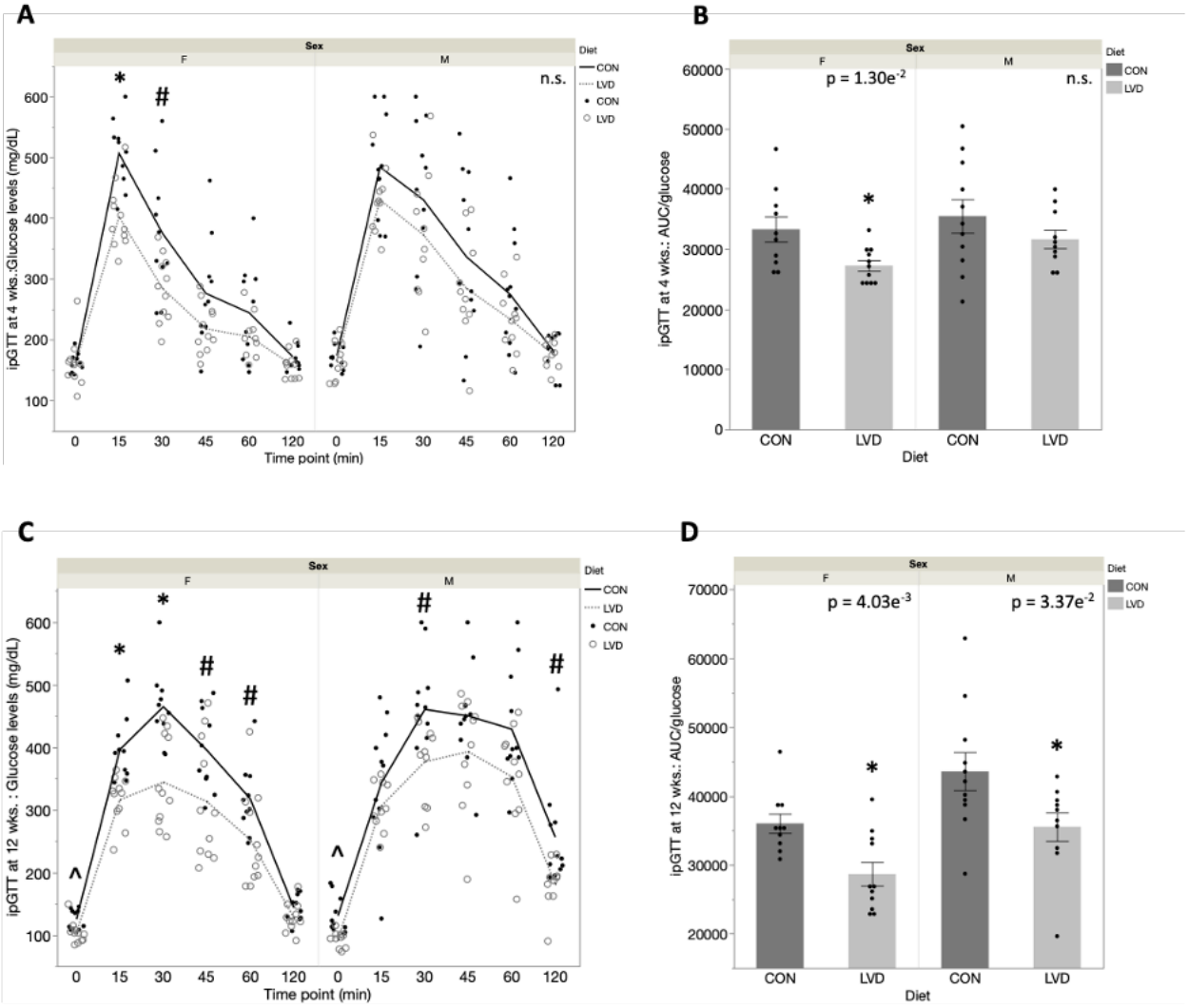


Figure 4. Effects of developmental vitamin D deficiency on CC051 G_1 offspring adiposity Each dot represents a single mouse. Data separated by diet and sex. (A) Percent lean & fat mass at 4 weeks of age and (B) 12 weeks of age. (C) Perigonadal white adipose tissue weight adjusted for body weight at 12-13 weeks of age.

Maternal dietary depletion of 25(OH)D_3 improves CC051 offspring glucose tolerance

Intraperitoneal glucose tolerance tests were performed on the G_1 offspring at two time points: 4 weeks of age (Weaning), and 12 weeks of age (Fig. 1a). At 4 weeks of age, the female G_1 LVD offspring exhibited a significant increase in glucose tolerance ($p = 1.30 \times 10^{-2}$, Fig 5b, AUC/glucose; $p = 3.7 \times 10^{-4}$, Fig. 5a, GTT at 15 min.; $p = 2.06 \times 10^{-2}$, Fig. 5a, GTT at 30 min.), while the male G_1 LVD offspring displayed a glucose tolerance similar to their male G_1 CON offspring counterparts (Figs. 5a and 5b). At 12 weeks of age, the female G_1 LVD offspring maintained significantly greater glucose tolerance over their CON counterparts ($p = 4.03 \times 10^{-3}$, Fig. 5d, AUC/glucose) with the male G1 LVD offspring exhibiting a significant increase in

glucose tolerance as well (p = 3.37 x 10^{-2}, Fig. 5d, AUC/glucose). Despite the difference in glucose tolerance, the LVD and CON G1 offspring had comparable levels of serum insulin in both males and females (Fig. 5e).



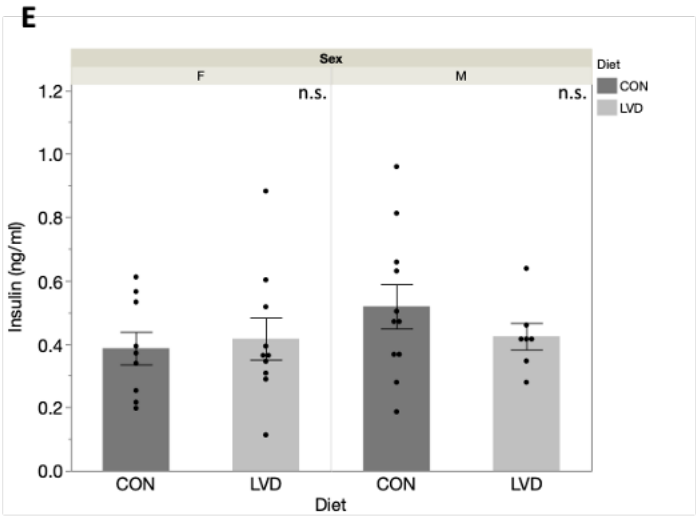


Figure 5. Effects of developmental vitamin D deficiency on CC051 G_1 offspring glycemic status Each dot represents a single mouse. Data separated by diet and sex. (A) ipGTT at 4 weeks of age. #p < 0.05, ^ p < 0.01, * p < 0.001. (B) AUC at 4 weeks of age. (C) ipGTT at 12 weeks of age. #p < 0.05, ^ p < 0.01, * p < 0.001. (D) AUC at 12 weeks of age. (E) Fasting insulin at 12-13 weeks of age.

Maternal dietary depletion of 25(OH)D_3 does not affect male reproductive organs

The male G1 offspring had their testes weighted and mature sperm quantified at 12-13 weeks of age. When adjusted for body weight, there was no significant difference between G1 CON and LVD for testes weight (Fig. 6a). There was also no significant difference in mature sperm counts (Fig. 6b) either between CON and LVD male G1 mice.

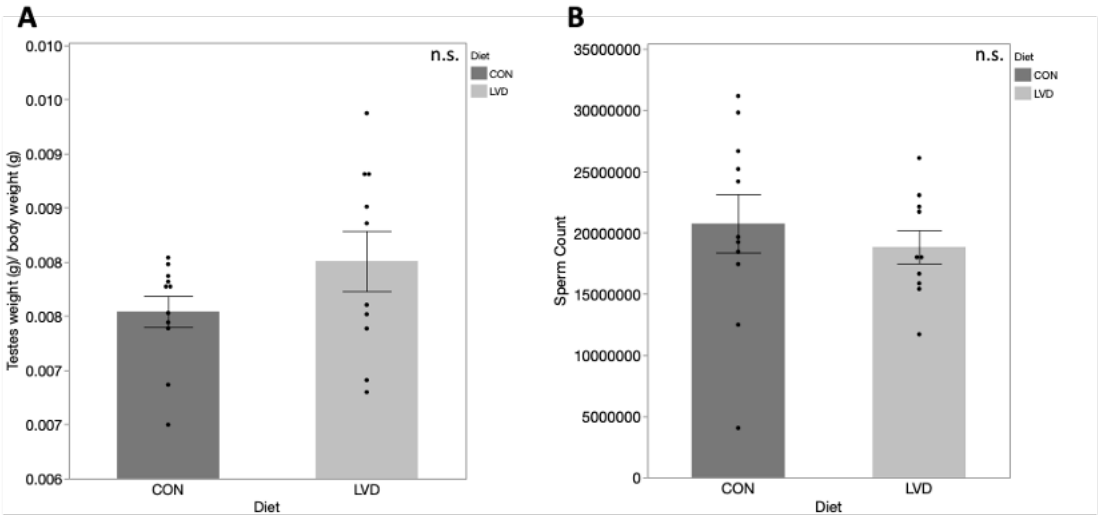


Figure 6. Effects of developmental vitamin D deficiency on CC051 male G_1 offspring testes and sperm Each dot represents a single mouse. Data separated by diet. (A) Testes weight adjusted by body weight at 12-13 weeks of age. (B) Mature sperm count at 12-13 weeks of age.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

In this study, we were able to access and characterize multiple phenotypes that arise due to dietary vitamin D deficiency during pregnancy, lactation, and development in the CC051 mouse strain.

We determined that while the LVD CC051 dams had significantly lower circulating sera 25(OH)D levels compared to the CON CC051 dams that it did not affect the LVD CC051 dams' serum calcium and serum insulin levels. A previous study has demonstrated that less than 4 weeks of dietary vitamin D depletion in sexually mature 8-wk-old female C57BL/6 mice can lead to deficiency[39], defined by the Institute of Medicine as having a serum 25(OH)D less than 50 nmol/L. Additionally, significant differences in serum calcium concentrations did not arise until the C57BL/6 mice had been on their vitamin D deficient diet that contain 5g/kg calcium for at least 12 weeks[39]. The lack of difference in serum calcium levels between the CC051 CON and LVD dams could be due to the higher levels of calcium, 11.63 g/kg, present in both diets used in our experiment. The fact that the LVD CC051 dams were not experiencing hypocalcemia could explain the similar fasting serum insulin levels with CON CC051 dams since calcium ions stimulate insulin release into the bloodstream[40-42]. We also found that dietary vitamin D deficiency did not result in any differences in growth rate or food intake in the CC051 dams. This could also be attributed to the LVD CC051 dams having adequate calcium intake to support normal, healthy bone growth and development.

The only area we identified a difference between the CON and LVD CC051 dams was in glucose regulation. The LVD dams exhibited improved glucose tolerance after just 6 weeks on diet compared to the CON dams. Another study also found that the glucose AUC for male mice on an LVD diet for 12 weeks was lower compared to the mice on the CON diet[43]. These results

are unexpected due to the association of vitamin D deficiency and glucose dysregulation seen in previous studies[44,45]. However, a previous study has shown that calcium supplementation during vitamin D deficiency normalizes glucose intolerance[46]. Taken together, the dietary vitamin D depletion did not negatively affect the CC051 dams' health during gestation and lactation of their offspring. Thus, any differing phenotypic effects observed between the CC051 CON and DVD offspring can be reasonably attributed to vitamin D deficiency rather than other negative maternal health outcomes.

The CC051 G_1 CON and LVD offspring did not display any differences in weight at birth. However, by the first week, the G_1 VDD mice began to grow at a slower rate than the G_1 CON mice. This difference in weight was maintained throughout development and into adulthood. DVD has been associated with low birth weight in humans, but many studies show that disparities in weight are resolved by 12 months of age due to 'catch-up' weight gain[46-50]. However, a previous mouse study has shown that DVD mice weighed less at weaning (21 days of age) than CON mice and another study has shown that vitamin D receptor knockout (VDRKO) mice weighed significantly less than WT mice at 2, 4, and 6 months of age[51-52].

The CC051 LVD G_1 mice were proportionally smaller in most ways measured by our study. When adjusted for body weight, VDR target organs, such as the heart, liver, and kidneys were not significantly different between the maternal diets. It has been shown that VDRKO mice do not have altered growth in these organs either[52]. The CC051 G_1 CON and LVD offspring also did not display any differences in the crown-to-rump distance at birth, which was in accord with the similar birth weights observed. A significant difference did arise in the male mice at 12-13 weeks of age, with the male LVD mice having a smaller crown-to-rump distance than their male CON counterparts. This difference in body length did not affect other body measurements, such as femur length and anogenital distance. Previous studies in both humans and animals have suggested that sufficient vitamin D levels are required for optimal fetal calcium and bone homeostasis[26,53-57]. However, a study of VDR null mice fetuses showed no gross skeletal

abnormalities, nor differences in length, mineralization patterning, and morphology of the long bones in the apical skeleton compared to WT mice. Thus, establishing that, with normal calcium levels, the vitamin D receptor is not essential for the control of placental calcium transfer in mice and, by extension, fetal mineral homeostasis[58]. A later study demonstrated that pregnant and lactating VDR null dams upregulate intestinal calcium absorption and lactate normally, thus providing adequate calcium needs for their offspring[59]. This difference in crown-to-rump distance between the male CC051 mice with different maternal diets is most likely not a result of impaired calcium homeostasis in skeletal development.

Developmental vitamin D deficiency did not affect CC051 male reproductive health, since there was no difference in testes weight (adjusted for body weight) and mature sperm count between the DVD and CON male mice. Vitamin D has previously been suggested to play a role in sperm development and function[60,61]. Vitamin D deficiency has also been shown to result in reduced sperm counts and lower fertility rates (female mice impregnated by vitamin D deficient males)[62], while VDRKO male mice have decreased sperm count and motility[63]. However, like fetal mineral homeostasis, these abnormal male reproductive phenotypes were due to hypocalcemia rather than being directly caused by vitamin D deficiency as these phenotypes were not observed in VDR null mice that were calcium sufficient [64-65].

Unlike the offspring of the LVD CC051 x CC041 reciprocal cross, the LVD CC051 G_1 mice did not differ significantly in lean and fat mass compared to CON CC051 G_1 mice. The weight of the LVD offspring's perigonadal white adipose tissue when adjusted for body weight also did not differ from the CON offspring (Fig. 7). Along with the LVD CC051 x CC041 reciprocal cross data, a previous study has found that dietary DVD was associated with increased adiposity[31]. Despite this result not being what we expected, a different study showed that for offspring born to either vitamin D sufficient (VDS) or vitamin D deficient (VDD) dams that the in-utero environment had no significant effect on total body fat percentage[66]. However, this study also found that the DVD mice did develop a higher percentage of visceral fat, which is

associated with metabolic dysfunction[66]. The difference in our results could potentially be because we measured body fat in the G_1 mice at 4 and 12 weeks of age, while the other studies did so at 19-24 weeks of age. Our LVD offspring mice may not have had a chance to develop increased adiposity or higher levels of visceral fat. Another factor could be that the CC051 mice are less genetically susceptible to DVD-induced adiposity or metabolic dysregulation than the strains used in the other studies[31,66].

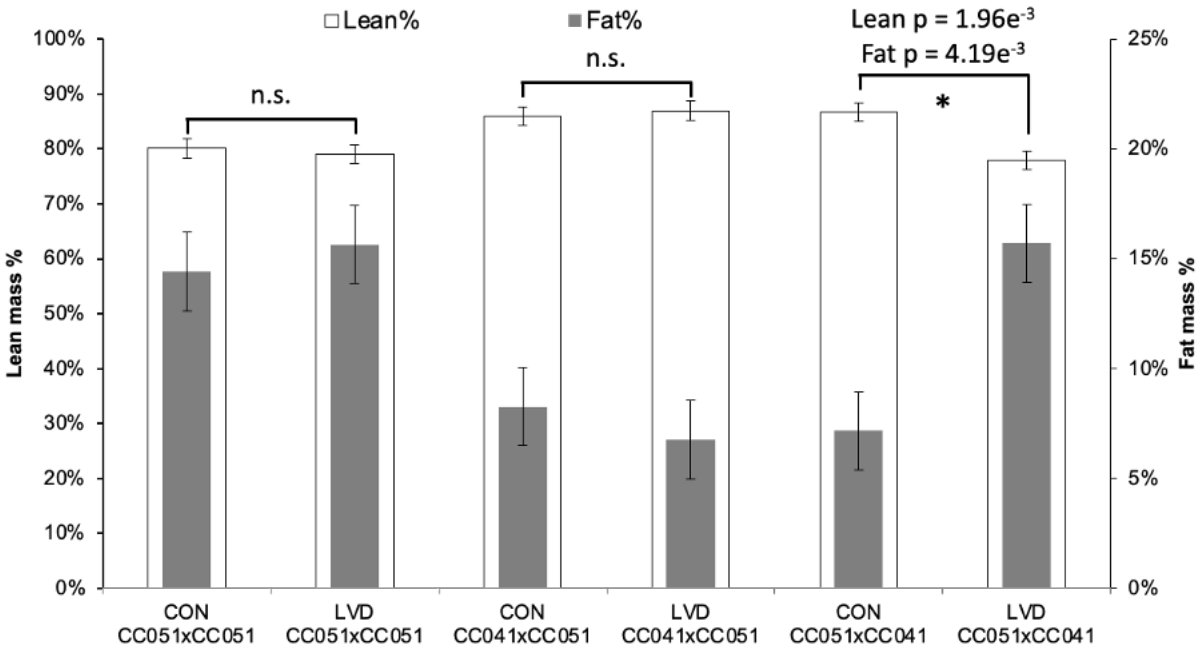


Figure 7. Effects of developmental vitamin D deficiency on male G_1 offspring adiposity Data separated by diet. Percent lean & fat mass at 12 weeks of age for CC051 and 8 weeks of age for CC041xCC051 and CC051xCC041

Similarly, to the LVD CC051 dams, the CC051 DVD offspring displayed an improved glucose tolerance at 12 weeks of age compared to the CON offspring. This trend of having a lower glucose AUC started for female DVD mice at 4 weeks of age, but wasn't exhibited by the DVD males until 12 weeks of age. Previous studies have shown that developmental vitamin D depletion led to glycemic regulation in the form of higher fasting glucose levels and GTT AUCs[27,31]. However, another study demonstrated that offspring born to a VDD dam showed no difference in baseline fasting glucose concentrations and glucose tolerance (AUC) at 15-16 weeks of age compared to offspring born to a VDS dam[66]. This finding in addition to our results

shows that in different strains of mice DVD does not result in lower glucose tolerance. As with the lack of DVD-induced adiposity seen in this strain, CC051 mice could potentially be more genetically resistant to DVD-induced glycemic dysregulation than previous strains researched.

Initially, the objective of this study was to characterize a non-genetically modified rodent model for DVD-induced adiposity. Instead, the DVD CC051 G_1 mice displayed post-natal growth retardation and improved glucose tolerance. This work adds support for the role of genetic individual variability in the response to vitamin D deficiency during development on adiposity and metabolic regulation[27, 31,66]. Thus, our findings highlight the importance of studying DVD in different CC mouse strains in order to deduce the potential genetic and epigenetic mechanisms behind the diverse phenotypic reactions to developmental vitamin D deficiency.

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